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THE ACID-SOLUBLE NUCLEOTIDES OF SALMON LIVER[1]

H. TSUYUKI, VIOLET M. CHANG, AND D. R. IDLER

Abstract

The acid-soluble nucleotides of spring salmon liver have been separated by anion-exchange chromatography at low temperature and characterized. Under these conditions the relatively labile uridine-5′-diphosphate nucleotides of acetylglucosamine, galactose, and glucuronic acid were obtained intact, a fact that is further substantiated by the complete absence of uridine-5′-diphosphate. The occurrence of these uridine diphosphate compounds and the absence of uridine diphosphate glucose is discussed in relation to the previously postulated role of inositol as a carbohydrate storage product. A new peptide-containing nucleotide, succinoadenosine-5′-phosphosulphate (peptide), was found in the fraction which immediately follows adenosine-5′-diphosphate. The parent base of this nucleotide, succinoadenine, was also isolated. The nucleotide pattern is simpler than that reported by other investigators for rat liver and wheat.

Introduction

Since the application of anion-exchange chromatography by Cohn (6, 7) to the separation of nucleotides, Hurlbert *et al.* (16) have applied this method to the separation of the acid-soluble nucleotides of rat liver. In recent years extensive work has been carried out on the identification and coenzymic role of nucleotides on a variety of tissues too numerous to review. However, very few published reports have appeared concerning the nucleotides in marine life. Jones and Murray (18) reported the presence of ATP,* ADP, AMP, DPN, IMP for rested and exhausted skeletal muscle of the codling. Storey and Love (36) partially characterized a succinoadenylic acid-like compound from cod liver, and Tsuyuki and Idler (39) identified a peptide of 6-succino-adenosine-5′-phosphosulphate in salmon liver.

In this study the acid-soluble nucleotide pattern of salmon liver will be presented and compared with the results obtained by other investigators. In addition, the advantages of low temperature chromatography employed in this study will be discussed.

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*The following abbreviations are used in this manuscript: UMP, uridine-5′-phosphate; UDP, uridine-5′-diphosphate; UTP, uridine-5′-triphosphate; AMP, adenosine-5′-phosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; IMP, inosine-5′-phosphate; IDP, inosine-5′-diphosphate; ITP, inosine-5′-triphosphate; GMP, guanosine-5′-phosphate; GDP, guanosine-5′-diphosphate; GTP, guanosine-5′-triphosphate; DPN, diphosphopyridine nucleotide; UDPAG, uridine-5′-diphosphate acetylglucosamine; UDPGAL, uridine-5′-diphosphate galactose; UDPGA, uridine-5′-diphosphate glucuronic acid; UDPX, uridine-5′-diphosphate derivative where X is a variable; P_t, total phosphorus; P_l, acid-labile phosphorus.

Methods

Preparation of Acid-soluble Fractions

The livers were excised from live spring salmon (*Oncorhynchus tschawytscha*) at sea and immediately frozen in dry ice. The powdered tissue was homogenized in a precooled Waring blendor with cold 0.6 *N* perchloric acid (1.5 ml. per g. of tissue) and centrifuged. The residue was extracted a second time with the same volume of cold 0.2 *N* perchloric acid. The supernatants were combined and neutralized with 5 *N* potassium hydroxide to pH 7.0. All operations were carried out at 0° C. The slightly soluble potassium perchlorate (0.75 g. per 100 ml. at 0° C.) began to precipitate immediately, and after standing a few hours the supernatant was decanted, lyophilized to one-quarter its volume, and a further crop of potassium perchlorate removed to reduce the salt concentration. The sample was chromatographed on Dowex-1 anion-exchange resin employing gradient elution exactly as described by Hurlbert *et al.* (16). The liver nucleotides were separated into fractions A to J by elution with formic acid. Each of these fractions was then further purified on Dowex-1, using an ammonium formate – formic acid solvent system.

Low Temperature Chromatography

When the nucleotide pattern from a column operated at room temperature according to Hurlbert *et al.* (16) was compared to that obtained at 0° C., the differences observed warranted the use of refrigeration throughout the chromatographic procedures. The Gilson fraction collector pan was insulated to minimize heat exchange and the refrigerant was circulated through the pan and the jacketed column at a rate sufficient to maintain the temperature of each at 0° C.

Analysis and Rechromatography

The nucleotide fractions were initially located by reading their optical densities at 260 mμ in a Beckman spectrophotometer. Subsequently, the nucleotides were located automatically by means of an ultraviolet recorder attached to the Gilson fraction collector. In this system a combination of filters permitted ultraviolet light in the region of 257 mμ to pass through a quartz cell through which the eluent passed. Thus only material absorbing in the 257 mμ region is recorded. When the results from several column runs obtained by the automatic ultraviolet recording device were compared to the readings from the Beckman DU, it was found that with the exception of a few very small components all major and minor peaks were detected.

Appropriate fractions of a peak were pooled and the solvents were removed by freeze-drying and the nucleotides analyzed or again chromatographed. The ammonium formate was removed by several methods; (*a*) by freeze-drying for several days; (*b*) by ion-exchange (IR-120) followed by freeze-drying; and (*c*) by freeze-drying followed by high vacuum (10^{-7} mm. Hg) sublimation for a few hours. The samples were then dissolved in water and aliquots taken for the various analyses.

Analytical Methods

Ultraviolet Light

The maximum and minimum ultraviolet absorption of the nucleotides in acid (pH 1–2) was read from a Beckman Model DK spectrophotometer recording. The nucleotide concentrations in acid were calculated from the appropriate extinction values compiled by the California Foundation for Biochemical Research. Adenine succinic acid was determined by absorption at 266 mμ, using $E = 16.9 \times 10^3$ at pH 1.0.

Phosphate

Inorganic phosphate was determined by the method of Lowry and Lopez (24). All recorded values for total and labile phosphate have been corrected for inorganic phosphorus. For labile phosphate the sample was hydrolyzed directly in 18×150 mm. calibrated colorimeter tubes, containing 4.0 ml. of 1 *N* sulphuric acid, for 15 minutes in a boiling water bath. The color was developed by the Fiske–Subbarow method (11) and read after 15 minutes in an Evelyn colorimeter with a 660 mμ filter. For total phosphorus the samples were brought to dryness in colorimeter tubes and digested with 1.0 ml. of 4 *N* perchloric acid for at least one hour at 160° C. (20). The tubes were then partially cooled, one drop of 30% hydrogen peroxide was added, and the contents were digested for an additional 40 minutes. The color was developed as for labile phosphate.

The position of the phosphate attached to the nucleotide sugar was determined by spraying the paper chromatograms with di-(*p*-dimethylaniline) methane reagent (41) as well as by use of the specific enzymes, 3′- and 5′-nucleotidase.

cis-Glycol and Sulphate

cis-Glycol was determined spectrophotometrically (9). Sulphate was determined colorimetrically after conversion to hydrogen sulphide (25), which was then reacted with *p*-phenylenediamine to form Lauth's violet (32).

Sugars

Ribose was determined according to Meijbaum (26). The ribose values of pyrimidine ribosides were used qualitatively. Uronic acid was determined by the Dische (8) method, reducing sugars by the Folin and Malmros (12) method, and N-acetylglucosamine by the method of Reissig *et al.* (33). In the Reissig assay the large difference in the extinction values enabled qualitative distinction between N-acetylglucosamine and N-acetylgalactosamine.

Amino Acid

The peptides were hydrolyzed in 6 *N* hydrochloric acid for 12 hours at 120° C. in sealed tubes. The acid was removed at 100° C. in a stream of nitrogen and the amino acids were determined quantitatively by the method of Moore and Stein (27).

Paper Chromatography

Whatman No. 1 and occasionally No. 3 papers were used ascending. Where it was necessary to elute the nucleotides for further analyses washed papers were used because of ultraviolet-absorbing contaminants in the paper. The papers were washed with a 1:1 mixture of ethanol and 1.2 *N* acetic acid, and rinsed thoroughly with water followed by ethanol according to the procedure of Kay *et al.* (19). The solvent systems used are indicated in the tables. The free bases and their derivatives were located by use of a mineralight lamp, the hexoses and uronic acid by aniline phthalate (31), hexosamines and acetylhexosamines by the modified Elson-Morgan reagent (30), amino acids by ninhydrin (22), and phosphorus by the Hanes and Isherwood method (14). Both the purine and pyrimidine nucleotides were hydrolyzed to their free bases by heating for 2 hours at 175° C. with 90% formic acid.

Results

A typical chromatogram of the acid-soluble nucleotides of salmon liver is shown in Fig. 1. Fractions A to J inclusive were present in every column that was analyzed. No fraction appeared beyond J even after prolonged elution. However, the relative amounts of each fraction showed considerable variation between individual salmon and between sexes. Each of the fractions has been analyzed and its principal components have been characterized.

The properties of fractions A, B, C, D, H, and J are summarized in Table I, together with the analytical data for their identification. Fraction A was separated into three components (A_1, A_2, and A_3) by column chromatography using ammonium formate as the developer. When each of these was subjected to further separation on paper, A_1 proved to be a single component, DPN, showing a typical cyanide reaction. A_2 was composed of two fractions, A_{2a} and A_{2b}, which were AMP and succinyl adenine respectively. These two components behave chromatographically alike in many systems but could be separated in *n*-butanol – acetic acid. A_3 was also separated into two components on paper. A_{3a} exhibited the ultraviolet absorption of uric acid. However, uricase did not reduce the absorption at 283 mμ and it was distinguishable from uric acid by paper chromatography using *n*-propanol – water (60:40). A_{3b} has not yet been identified.

Peak B proved to be a single component, GMP.

After a second column purification fraction C was separated into six components (Fig. 2). However, during the initial stages of this investigation, when the ion-exchange procedure was carried out at room temperature, it is significant that only two components (UMP and IMP) were obtained. Presumably the less stable remaining components were destroyed during column chromatography. The first component, C_1, proved to be hypoxanthine. When the known hypoxanthine derivatives IMP, IDP, and ITP were treated with 4 *N* formic acid for 3 days at room temperature (conditions considerably more drastic than the chromatographic procedures) there was no evidence of free hypoxanthine. At present there is no explanation for the presence of

hypoxanthine in this fraction except as a product of the partial decomposition of one of the C fractions C_4, C_5, or C_6. Components C_2 and C_3 were shown to be UMP and IMP respectively. C_4 consists of the bases uracil and hypoxanthine. The identification of C_5 and C_6 is continuing.

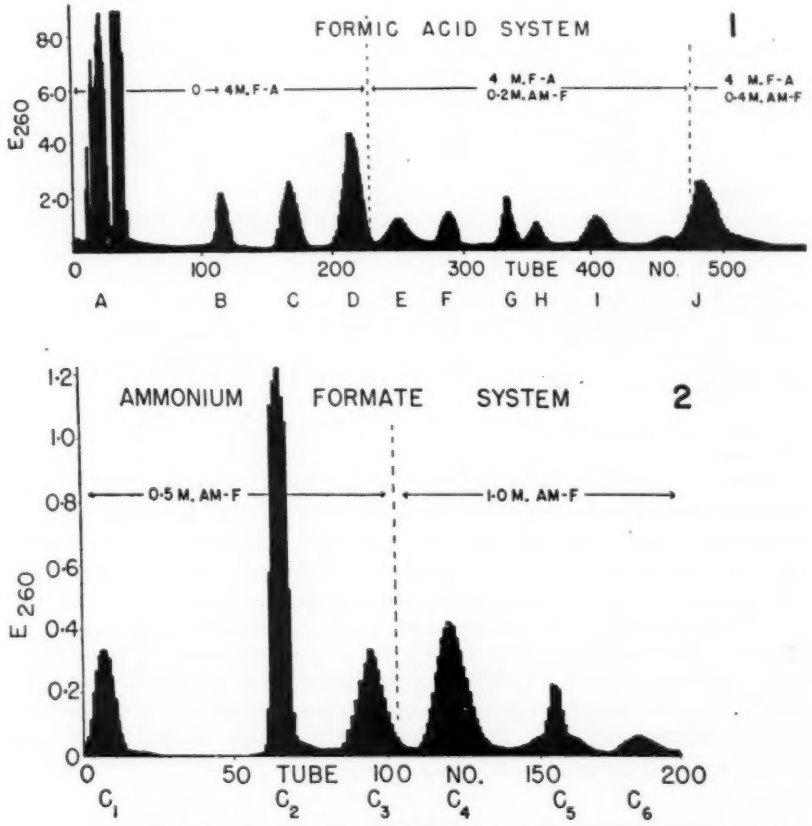


FIG. 1. Chromatography of the perchloric acid extract from 125 g. of liver using the formic acid system. The nucleotides were separated by gradient elution on a 1.7×28 cm. bed of Dowex-1 formate resin at 0° C. The mixing volume was 2 l. and 10 ml. fractions were collected. The optical densities of the eluted fractions at 260 mμ are plotted for each chromatographic fraction. The symbol F.A. refers to formic acid and AM-F to ammonium formate.

FIG. 2. Rechromatography of fraction C. Fraction C obtained from the formic acid system was rechromatographed on a 1×20 cm. bed of Dowex-1 formate resin at 0° C. The mixing volume was 500 ml. and 5.0 ml. fractions were collected. The column was developed with an ammonium formate solution.

TABLE I

ANALYSES OF FRACTIONS A, B, C, D, H, AND J

Fraction	Components	Chromatographic properties similar to:*	Spectra in acid		Per mole			Base*
			λ max.	λ min.	P_f	P_i	Ribose	
A	A_1	DPN[1,7]	256	236	1.82	0		Adenine[4,6]
	A_{1a}	AMP[1,7]	257	230	0.88	0	0.91	Adenine[4,6]
	A_{1b}	Succinoadenine[1,7]	270	231	0	–		
	A_{1a}'		283		0			
	A_{1b}'		257					
B	B_a	GMP[1,7]	257	228	1.04	0	1.20	Guanine[4,6]
C	C_1	Hypoxanthine[1,4,6]	248	215	–	–	–	Hypoxanthine[1,4,6]
	C_2	UMP[1,7,9]	261	230	0.93	0	+	Uracil[4,6]
	C_3	IMP[1,7,9]	248	224	1.04	0	1.0	Hypoxanthine[1,4,6]
	C_4							Uracil and hypoxanthine
	C_5		256	231				Adenine[4,6]
D	D	ADP[1,6]	257	231	2.0	0.92	1.0	Adenine[1,4,6]
H	H	GDP[1,6]	257	230	2.0	1.06	—	Guanine[4,5]
J	J	ATP[1,6]	257	236	3.1	2.2	–	Adenine[4,6]

*Superscripts refer to solvent systems used as follows:
(1) Isobutyric acid/conc. ammonium hydroxide/water, 66/1/33; (2) ethanol/1 *M* ammonium acetate (pH 3.8), 7.5/3; (3) 0.1 *M* sodium phosphate, pH 6.8/ammonium sulphate/*n*-propanol, 100/60/2; (4) aqueous ammonia, pH 10.0; (5) *n*-butanol/water/formic acid, 77/13/10; (6) water-saturated *n*-butanol/ammonia, 100/1; (7) *n*-butanol/acetic acid/water, 2/1/1; (8) isobutyric acid/conc. ammonium hydroxide/water, 57/4/39; (9) ethanol/1 *M* ammonium acetate (pH 4.5), 65/35; (10) ethyl acetate/pyridine/ammonium hydroxide/water, 40/20/12/12, paper treated with boric acid; (11) *n*-butanol/acetic acid/water (boric acid saturated), 4/1/5; (12) *n*-butanol/pyridine/water, 45/25/40.

Fraction D consisted solely of ADP, H of GDP, and J of ATP (Table I).

Fractions F, G, and I are all derived from UDP and have been identified as UDPAG, UDPGAL, and UDPGA respectively. Whereas UDPAG has been isolated and characterized from yeast (29, 3), animal liver (28, 15), hen oviduct (37), mung bean seedlings (34), and UDPGAL from mung bean seedlings (13), and UDPGA in rabbit liver (35), and mung bean seedlings (34), their presence in marine forms of life has not yet been reported. The analytical data on which the identification of F, G, and I is based appear in Table II. The characterization of UDPAG is based on a molar ratio of base: P_f:P_i: reducing value: N-acetylglucosamine of 1:2:1:1:1. N-Acetylglucosamine was identified by paper chromatography and by colorimetric assay (33), and differs from N-acetylgalactosamine by having an extinction value about three times greater at 585 mμ.

TABLE II

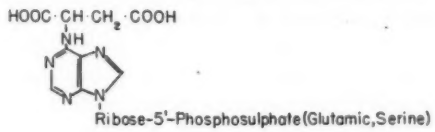
PROPERTIES OF UDPX COMPOUNDS IN SALMON LIVER

Fraction*	Chromatographic properties similar to†	Base†	Per mole			Reducing value	Uronic acid	N-Acetyl glucosamine	X component†
			P_f	P_i	Ribose				
F	UDPAG[1,3,8]	Uracil[4,6]	1.88	0.92	+	1.08		1.05	N-Acetyl-glucosamine[2,10]
G	UDPGAL[1,3,8]	Uracil[4,5]	2.05	0.92	+	1.0			Galactose[11,12]
I	UDPGA	Uracil[4,5]	1.97	1.01	+	+	0.86		Glucuronic[7,9] acid

*All three fractions showed a uridine type spectrum.
†Superscripts refer to solvent systems in Table I.

Fraction G was identified as UDPGAL by virtue of equimolar proportions of UDP and reducing sugar. The sugar was shown to be galactose on several paper chromatograms. Fraction I was likewise shown to be UDPGA by a UDP to uronic acid ratio of approximately unity. Glucuronic acid was distinguished from galacturonic acid by paper chromatography and by the ease with which the former compound lactonizes (30).

A hitherto unknown nucleotide, of which a preliminary account has appeared (39), was found in the E fraction, which immediately follows ADP in the chromatographic picture (Fig. 1). This fraction was separable into three components (E_1, E_2, and E_3) by paper chromatography. Compound I (E_2) moved between the other two and generally comprised approximately 80% of the fraction. The minor components E_1 and E_3 are still being investigated. Analytical data on I show an approximately equimolar ratio of succinoadenine, total P, ribose, sulphate, and *cis*-glycol (Table III). A peptide consisting of glutamic acid and serine was found to be associated with this compound. On the basis of these analyses and partial degradation studies (39) the structure indicated below has been tentatively assigned to this compound.



Compound I

TABLE III

ANALYSIS OF COMPOUND I

	Moles
Succinoadenine*	1.00
Ribose	1.12
Labile P	0.00
Total P	1.05
Sulphate	1.37
cis-Glycol	1.27

*Arbitrarily chosen as 1 mole.

The occurrence of either succinoadenine or succinoadenylic acid has been reported in yeast (4), *Penicillium chrysogenum* (1), *Escherichia coli* (23), mammalian liver (17), and cod liver (36). The lability of compound I suggests the possibility that succinoadenylic acid isolated from other natural products by ion-exchange chromatography at room temperature is a decomposition product from a larger molecule. The apparent absence of adenyl succinic acid in the present study strengthens this belief. The occurrence of succinoadenine in fraction A appears to be in disagreement with Joklik's statement (17) that it behaves chromatographically similar to succinoadenylic acid.

Discussion

The distribution of acid-soluble nucleotides in fish liver is different from that of other tissues. Whereas in rat liver (16) and in wheat plants (2) the mono-, di-, and tri-phosphates of cytidine are present, these were not detected in the fish liver, nor were GTP, UDP, and UTP. The nucleotide picture appears to tend towards simplicity compared to that of the other two tissues. The absence of UDP, which should appear in the region of fractions F, G, and I, is significant. This nucleotide was not detected, though it was searched for specifically. It would appear then that unless low temperature column chromatography is employed UDP appears as a decomposition product of relatively labile UDPX compounds.

The role of UDPX compounds is interesting to consider. Fischer (10) hypothesized that the presence of relatively large amounts of inositol in shark fins and the absence of glycogen and other reserve carbohydrates in the liver might mean that inositol may function as a source of blood sugar by the re-opening of the inositol ring. Recently, in his biochemical studies on inositol, Charalampous (5) discovered an enzyme system from rat kidney which catalyzed the oxidative conversion of inositol to glucuronic acid through the postulated mediation of UDP glucose followed by UDPGA. An analysis of a number of fish livers in this laboratory showed that there is in this tissue a set of conditions suggestive of the role of inositol as a carbohydrate storage product. Inositol analyses from a number of tissues from salmon, including the liver, indicate the presence of this compound at relatively high levels (40). The presence of reducing sugars in fish tissue appears to be a post-mortem phenomenon; these compounds do not appear to be present in fresh fish (38). UDPGAL and UDPGA were found to be present in significant concentrations in the liver, whereas UDP glucose was found to be absent. The reversible conversion of UDP glucose to UDPGAL has been demonstrated by Leloir (21) in *S. fragilis*. UDPGAL and UDPGA, therefore, might be implicated in the conversion of inositol to glucuronic acid in addition to other coenzymic roles in which these nucleotides are involved.

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PHARMACOLOGICAL STUDIES ON NEW SYNTHETIC ANTITUSSIVE AGENTS[1]

C. I. Chappel, M.-G. P. Stegen, and G. A. Grant

Abstract

In an attempt to overcome the inherent disadvantage of narcotic antitussive therapy, three basic alkoxy-alkyl esters of phenothiazine-10-carboxylic acid were synthesized and tested for pharmacological activity. These compounds possessed antitussive activity in the cat in the range of activity of codeine. Dimethyl-amino-ethoxy-ethyl phenothiazine-10-carboxylate was chosen, on the basis of strong antitussive activity coupled with low acute toxicity and low antispasmodic activity, for chronic toxicity studies and clinical trial. This compound is devoid of central depressant or analgesic properties and possesses a moderate local anaesthetic action.

Introduction

Numerous side effects have been described with the use of narcotics in antitussive therapy. They include tolerance and addiction, respiratory depression, toxic and allergic reactions. These well known findings have prompted the search in this laboratory for effective synthetic non-narcotic substitutes for codeine which might be free of these liabilities. Recently von Seemann (12) synthesized a series of esters of phenothiazine-10-carboxylic acid. This paper describes the pharmacological study of several basic alkoxy-alkyl esters from this series. These compounds were compared in respect to antitussive activity, antispasmodic and local anaesthetic activity, and acute toxicity. More extensive pharmacological and toxicological studies were conducted on dimethyl-amino-ethoxy-ethyl phenothiazine-10-carboxylate.

Methods

The antitussive activity was studied following the method of Domenjoz (3). Mature cats were lightly anaesthetized with sodium pentobarbital 30 mg./kg. intraperitoneally; more was administered intravenously during the period of the tests if it was required. Deep anaesthesia was avoided as this inhibited the cough reflex. Through an incision parallel to the jugular furrow, the superior laryngeal nerve was dissected free at the point where it emerges from the larynx. By means of a bipolar platinum electrode, square wave stimuli of five pulses per second and 5 milliseconds' duration were applied to the laryngeal nerve for 1/5 minute or until a clear response was obtained. The stimuli were not applied for longer than 1/2 minute. The pulse potential was adjusted between 1 and 5 volts so as to evoke a series of inspiratory gasps or "coughs" which were recorded on a revolving drum by means of a writing lever attached to the abdominal muscles just below the sternum. The blood pressure was recorded simultaneously through the femoral artery using an ink-writing mercury manometer.

[1] Manuscript received November 5, 1957.
Contribution from Ayerst, McKenna & Harrison, Ltd., Montreal.

After a 30 minute rest period, stimuli were applied to the nerve and the pulse potential was adjusted to produce three or four consecutive coughs of equal intensity. The test compound was then given intravenously or by oral gavage, and electrical stimulation was applied to the nerve every 5 minutes until the responses obtained returned to the pretreatment level. The per cent inhibition of cough responses was measured directly on the kymograph paper by comparing the height of deflections obtained before and after treatment. A second dose of the same compound was not given to the preparation until the effect of the first dose had ceased and at least three responses of pretreatment level were obtained.

The three phenothiazine derivatives were studied for antispasmodic activity using isolated strips of guinea pig ileum and for their effect on the passage of a test meal from the stomach of the rat. The guinea pig ileum was suspended in oxygenated Locke's solution at 37° C. Test compounds were added to the bath 2 minutes prior to the addition of barium chloride (100 μg./ml.). The effective dose$_{50}$ (ED_{50}) was obtained from four or five point log dose – response curves using the method of Litchfield and Wilcoxon (7). Rats weighing 140–150 g. and fasted for 24 hours were used in the tests for measuring the gastric emptying rate. The compounds were administered, by stomach tube, to groups of eight animals 40 minutes before a test meal of 2 ml. of a thick suspension of barium sulphate (2 g. in 1 ml. of water) also given by gavage. Ninety minutes later the animals were sacrificed by stunning. The stomach was removed from the body and the contents were washed into test tubes and centrifuged. The amount of barium sulphate remaining in the stomach was read directly from the centrifuge tube. The mean percentage of the test meal remaining in the stomach at each of four doses of the test compounds was plotted on a logarithmic scale. The ED_{50}, or dose which inhibits the passage of 50% of a test meal from the stomach, and the standard error were obtained from the regression lines (7).

The local anaesthetic activity was measured in the rabbit eye by the method of Luduena and Hoppe (8). A blunt glass rod touched lightly to the cornea was used to evoke the wink reflex. Anaesthetic activity is expressed as the TAC_5, or concentration producing anaesthesia for 5 minutes. The acute toxicity values were obtained by oral administration of the compounds to the mouse and rat. Three or four doses of each compound were administered to groups of 10 animals. The mortality present at 24 hours was recorded and the lethal dose$_{50}$ (LD_{50}) and standard error calculated (7).

Additional pharmacological studies were done on dimethyl-amino-ethoxy-ethyl phenothiazine-10-carboxylate. Anticholinergic activity was measured against spasms induced in isolated strips of guinea pig ileum by the addition of acetylcholine (0.1 μg./ml.). The effect on gastric secretion was determined in rats operated by the method of Shay *et al.* (9). Sedative tests in the rat were done by the method of Chappel *et al.* (1), and analgesic tests by the method of D'Amour and Smith in the mouse (2). Hypnotic potentiation was determined by the prolongation of sleeping time of mice injected intraperitoneally with 50 mg./kg. of sodium pentobarbital. The effect on blood

pressure and respiratory function was studied by intravenous injection to dogs anaesthetized with sodium pentobarbital. Blood pressure was recorded from the femoral artery with a Sanborn Electromanometer,* and respiratory function with a Peck and Waller automatic tidal volume recorder.†

Chronic toxicity studies were done in rats and dogs. Groups of 20 rats of each sex were fed 0.1, 0.06, and 0.04% of the test compound in a commercial rat diet for a period of 32 weeks. The food intake of these animals was recorded each day, and the intake of test drug calculated to be 45–75, 25–40, and 15–30 mg./kg. daily, in the respective groups. Body weights were recorded weekly and hematological studies were done on four animals from each group once a month. At the end of the study the animals were sacrificed and their organs were weighed, examined for gross pathology, and prepared for histological examination.

Two mature mongrel dogs, one male and one female, were given 50 mg./kg. of the test drug daily for 1 year. The compound was administered orally in capsule form. Body weights were recorded weekly and hematological studies and pathological examinations were conducted as described for the rat.

Results and Discussion

Several techniques for producing experimental cough in animals were studied in order to find a reproducible test method for the assay of antitussive activity. Höglund and Michäelsson (4) elicited coughs from unanaesthetized guinea pigs by exposure of the animals to ammonia vapor. We found a wide variation in the response of animals to repeated exposure to this irritant. The depression of coughs after these animals were treated with codeine was often within the range of variation encountered in untreated animals. Stefko and Benson (10) produced cough in dogs by stimulation of the mucosa of the trachea through embedded electrodes. This method has the decided advantage of allowing the use of unanaesthetized animals, and after a short recovery period from the surgery, the cough responses on stimulation were satisfactory. However, three animals which we prepared gradually became refractory to the stimulus. At necropsy, the stainless steel electrodes were found to be encapsulated by fibrous tissue; we attributed the decrease in response to the tissue reaction.

In our hands the method of Domenjoz (3) proved most satisfactory and our results with codeine are in reasonable agreement with those obtained by Toner and Macko (11) and Levis (6), who used the same method. Toner and Macko (11) found that 1 mg./kg. of codeine injected intravenously suppressed cough in 80% of the animals tested, and Levis (6) obtained a 70% inhibition of cough with an intravenous dose of 1.5 mg./kg. of codeine. Although Toner and Macko (11) did not state clearly their criterion of cough inhibition, ours was the same as that used by Levis (6). Such close agreement for data obtained in different laboratories would appear to emphasize the reliability of this test for antitussive activity.

* Sanborn Company, Cambridge, Mass., U.S.A.
† Phipps & Bird Inc., Richmond, Va., U.S.A.

The general formula for these compounds is shown in Fig. 1. R-groups in the compounds studied have been methyl, ethyl, or isopropyl. The antitussive activity as measured by the number of cats which showed a depression of cough response by 50% or more for at least 5 minutes at each dose administered appears in Table I. The percentage of inhibition was measured directly on the kymograph paper by comparing the height of the deflections obtained during a "cough response" before and after treatment. Although pretreatment responses were obtained before another dose was administered, the possibility of summation of subthreshold effect of the drug and of the following dose could not be ruled out.

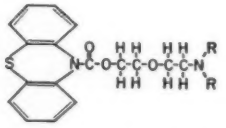

FIG. 1. General formula.

TABLE I

ANTITUSSIVE ACTIVITY

Compound	R	Salt	No. cats per group	Dose, mg./kg. I.V. 0.25	0.5	1.0	2.0	4.0	Approx. ED_{50}, mg./kg.
				Fraction of group showing 50% or greater suppression of cough					
I	$-CH_3$	HCl	8	–	0/8	2/8	5/8	7/8	1.6
II	$-CH_2CH_3$	HCl	8	0/8	1/8	7/8	8/8	—	0.7
III	$-CH(CH_3)_2$	HCl	8	0/8	3/8	8/8	—	—	0.6
Codeine		H_3PO_4	5	—	0/5	2/5	4/5	5/5	1.2

It will be noted from Table I, by comparing the approximate ED_{50}, that the test compounds possess antitussive activity in the same range as that of codeine. It is also evident that activity increases with increasing size in the alkyl group from methyl to isopropyl. It will be seen from Table II that this same relationship held with respect to antispasmodic activity, effect on gastric emptying rate, anaesthetic potency, and acute toxicity.

The relative antispasmodic activities of the phenothiazine derivatives as tested *in vitro* parallel their effect on the gastric emptying rate in the rat. In both cases the methyl derivative is the least active and the isopropyl derivative the most active—Fig. 2. Although their modes of action are different, both the phenothiazine derivatives and codeine delayed the passage of gastric content. The log dose – response curves so obtained were not parallel. Nevertheless, at all the doses tested the methyl phenothiazine derivative had less effect than codeine.

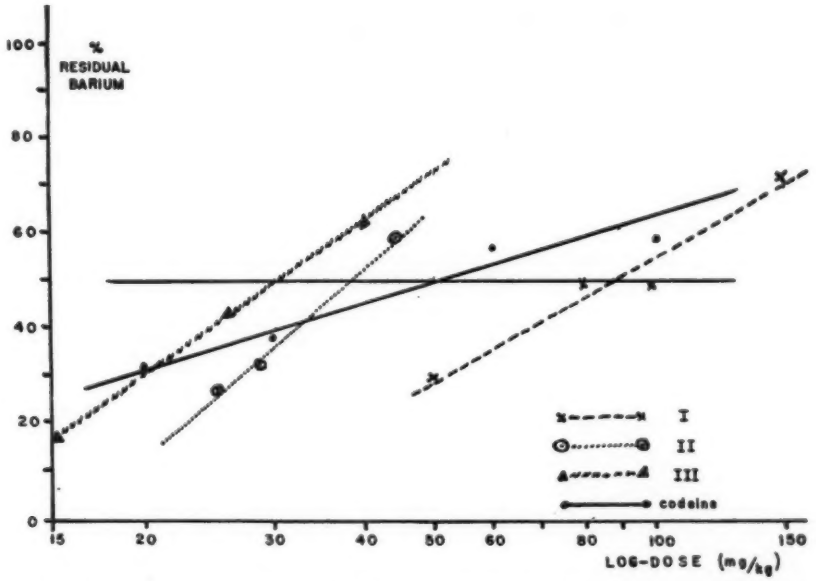

FIG. 2. Comparison of oral action of compounds I methyl, II ethyl, and III isopropyl dialkyl derivatives of basic alkoxy-alkanol esters of phenothiazine-10-carboxylate with codeine on the gastric emptying rate in rats.

Because of its low acute toxicity and low antispasmodic activity, the methyl derivative was chosen for further pharmacological study and clinical trial. This compound, at an intravenous dose of 1.5 mg./kg. to 10 cats and at an oral dose of 7.5 mg./kg. to three cats, produced complete suppression of cough responses lasting an average of 40 and 80 minutes respectively. It was devoid of anticholinergic or antihistaminic activity when tested against acetylcholine- or histamine-induced spasms of isolated guinea pig ileum. When tested by the Shay (9) procedure, a subcutaneous dose of 10 mg./kg. had no effect on the volume or acidity of the gastric juice of the rat.

At a dose of 25 mg./kg. given subcutaneously, the methyl derivative failed to influence the spontaneous activity of the rat and the same dose in the mouse did not affect the sleep time after sodium pentobarbital. When tested by the method of D'Amour and Smith (2) for analgesic properties, no activity was found at a subcutaneous dose of 25 mg./kg.

Given intravenously to dogs anaesthetized with sodium pentobarbital, 2 mg./kg. of the methyl derivative did not cause any significant effect on the blood pressure or on the respiratory rate. Doses of 2–8 mg./kg. caused a transient fall in blood pressure lasting 5–15 minutes. This effect was accompanied by a slight tachycardia and tachypnoea, both of which returned to normal with the rise of blood pressure.

In the acute toxicity tests (Table II), the methyl derivative was found to be equal in toxicity to codeine in the mouse and less toxic in the rat. In the

chronic toxicity tests in the rat no evidence of toxicity was observed. The body weight curves which do not differ significantly from control to treated groups are shown in Fig. 3. Monthly white and red blood cell counts, hemoglobin and hematocrit determinations were within normal limits for the rat. At necropsy no difference was seen in the weights of tissues from control and treated animals and both gross and microscopic examination of body tissues failed to show pathological lesions.

TABLE II

ACTIVITY AND TOXICITY OF PHENOTHIAZINE DERIVATIVES AND OF CODEINE

Compound	R	Antispasmodic activity G.P. ileum ED_{50}, μg./cc.	Inhibition of gastric emptying rate in the rat ED_{50}, mg./kg.	Local anaesthetic activity rabbit cornea T.A.C.$_{0.5}$,%	Oral acute toxicity LD_{50}, mg./kg.	
					Mouse	Rat
I	$-CH_3$	2.3±0.3*	89±7.3*	0.036	580±15*	1500±88*
II	$-CH_2-CH_3$	1.0±0.2	38±4.6	0.018	150±15	290± 8.13
III	$-CH(CH_3)_2$	0.7±0.04	31±2.5	0.007	94± 8	175±40
Codeine			50±7.3	Inactive at 5	600±73	615±30

* Standard error.

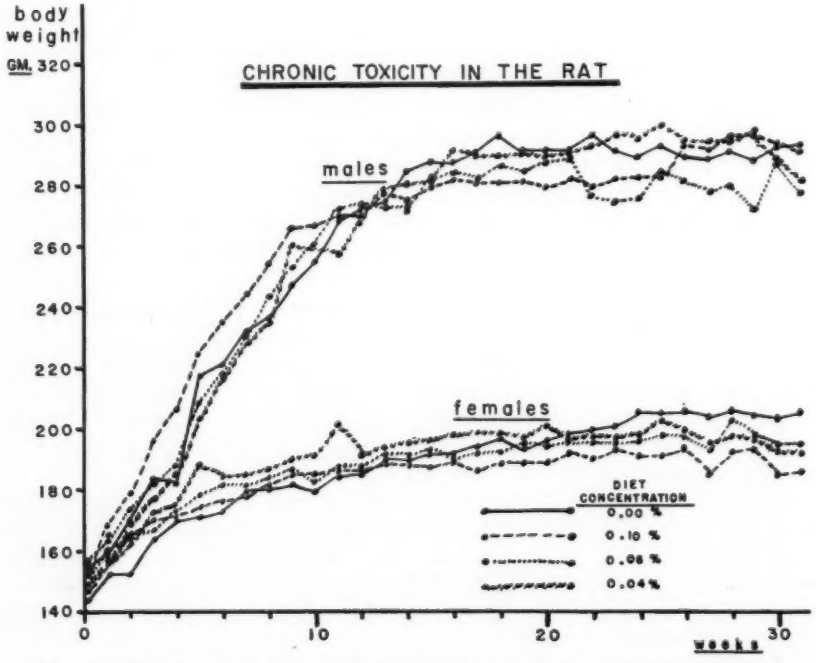

FIG. 3. Chronic toxicity of dimethyl-amino-ethoxy-ethyl phenothiazine-10-carboxylate in the rat.

The two mongrel dogs treated with 50 mg./kg. of the methyl derivative for 1 year showed no sign of toxicity. Both animals gained weight and were in good health during the study. Their hemograms, done every month, remained within normal limits, and at autopsy there was no evidence of gross pathology.

Clinical trials of this compound have been conducted (5). These studies have confirmed our findings in principle. A single dose of 25 mg. orally in the human causes an antitussive effect which is rapid in onset and lasts from 3 to 7 hours. In these trials no toxic or side effects have been noted.

Acknowledgments

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INSULIN, MAST CELL COMPONENTS, AND "ANAPHYLACTOID" INFLAMMATION[1]

V. W. ADAMKIEWICZ AND Y. L. LANGLOIS[2]

Abstract

The treatment of rats with an antihistaminic, or a long pretreatment with a histamine depletor, inhibits the "anaphylactoid" inflammation, and inhibits the sensitizing effect of insulin on this inflammation. However, the systemic administration of histamine, serotonin, and heparin does not restore the inhibited "anaphylactoid" inflammation, and does not restore the sensitizing effect of insulin. The three substances administered systemically are also unable to imitate, in normal rats, the sensitizing effect of insulin.

Introduction

We reported previously that insulin sensitizes the rat to the "anaphylactoid" inflammation produced by dextran (1). The same is true for inflammation produced by egg white or soluble glycogen (unpublished).

It is known that insulin administration markedly increases the secretion of gastric juice (12). The increase can be reproduced by an administration of histamine (3). It is also known that insulin causes a discharge of adrenaline and noradrenaline from the adrenal medulla (11). Again this effect can be imitated by injection of histamine (11).

Goth *et al.* reported recently (8) that the administration of dextran to alloxan diabetic rats is followed by a small rise in plasma histamine. But, if the diabetic rats are treated with insulin, the injection of dextran is followed by a much greater rise in plasma histamine.

These observations indicate that a certain relationship might exist between physiologic manifestations of insulin and histamine.

In the present study, we investigated whether the sensitizing effect of insulin on "anaphylactoid" inflammation is mediated through histamine. At the same time we tested serotonin (5) and heparin (4) which, like histamine (4), are present in the mast cells of the rat.

Methods

We used male Sprague–Dawley rats, 100–180 g., maintained in an animal house on Purina Fox Chow cubes and water.

Dextran, 6% w/v in saline (Abbott), was injected either intraperitoneally (i.p.) or subcutaneously (s.c.). The dose was 1 ml. (60 mg.) per rat, except in experiments V and VI. In experiment V the dose was 0.5 ml. (30 mg.) per rat. In experiment VI the dextran solution was diluted with saline to 0.005% w/v; 0.1 ml. of this (5 μg.) was injected s.c. per rat.

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Contribution from the Département de Physiologie, Faculté de Médecine, Université de Montréal, Montreal, Quebec. This work was supported by grant No. MA 640 received from the Canadian National Research Council.
[2]Fellow of the Lederle Medical Students' Research Fellowship.

Insulin–Zn, "Toronto" (Connaught) was injected s.c. The dose was four units (0.1 ml. commercial solution) per rat. In experiment VI the commercial insulin solution was diluted with saline to contain 0.005% w/v protein. Of this 0.1 ml. (5 μg.) was injected s.c. per rat.

Phenergan (Promethazine HCl, Poulenc) was dissolved in saline at 1% w/v. Of this, 0.2 ml. (2 mg.) was injected per rat intramuscularly (i.m.) into the thigh.

Compound 48:80 (*p*-methoxyphenethyl methylamine – formaldehyde polydispersed condensation product, Burroughs-Wellcome and Co.) was dissolved in saline at 0.1% w/v. The rats were injected according to the following schedule. On the first day, 100 μg. (0.1 ml.) were administered i.p. per rat. The dose was increased by 50 μg. (0.05 ml.) daily, until 500 μg. per rat was reached. Then the drug was administered s.c. and the dose was increased by 100 μg. daily until 1000 μg. per rat was reached. The dose of 1000 μg. s.c. per rat was continued until the 25th day.

Histamine phosphate (Nutritional Biochemicals) was dissolved in saline at 1% w/v. Of this, 0.1 ml. (1 mg.) was injected i.p. per rat. In experiment VI, histamine was prepared in saline solution at 0.005% w/v; 0.1 ml. (5 μg.) was injected s.c. per rat.

Serotonin (5-hydroxytryptamine creatinine sulphate, Sigma Chemical Co.) was dissolved in saline at 0.1% w/v; 0.1 ml. (100 μg.) was injected i.p. per rat.

Heparin (sodium salt, Connaught) was prepared in a 1% w/v saline solution; 0.1 ml. (1 mg.) was injected i.p. per rat.

A mixture was also prepared containing 1 mg. histamine, 1 mg. heparin, and 100 μg. serotonin per 0.1 ml. saline solution. This was injected i.p. once or every half hour, at the dose of 0.1 ml. per rat per injection.

Evans Blue (National Aniline) 0.5 ml. 1% w/v saline solution, was injected into the jugular vein under ether anaesthesia.

The degree of general "anaphylactoid" swelling was measured in ml. by dipping the left hind paw of the rat periodically into a volumetric apparatus (2).

All injections were done with No. 27 hypodermic needles and "Tuberculin" syringes. The animals were weighed regularly. The results were analyzed statistically (6, 7).

Results

I. Inhibition, by an Antihistaminic, of the Insulin Sensitization to "Anaphylactoid" Inflammation

Thirty rats, 93–115 g., were divided into three groups of 10. Group I control rats were injected with 1 ml. dextran s.c. on the back. Dextran injected at this site rarely produces an acute "anaphylactoid" inflammation. Group II rats received the same injection of dextran, plus four units insulin. Insulin sensitizes to "anaphylactoid" inflammation, so that rats of this group should now become inflamed. Group III received the same injections of dextran and insulin, plus 2 mg. phenergan.

Fig. 1 illustrates the results. In the Group I control rats, where dextran alone was injected s.c. on the back. the general inflammation occurred late

and was of weak intensity. Rats of Group II, which received insulin as well as dextran, showed early a marked inflammation (p, Groups I/II at 1, 2, and 3 hours, < 0.01). In Group III the administration of phenergan completely abolished the sensitization effect of insulin. These rats did not become inflamed (p, Groups II/III at 1, 2, 3, and 4 hours, < 0.01; p, Groups II/I at 1, 2, 3, and 4 hours, > 0.9).

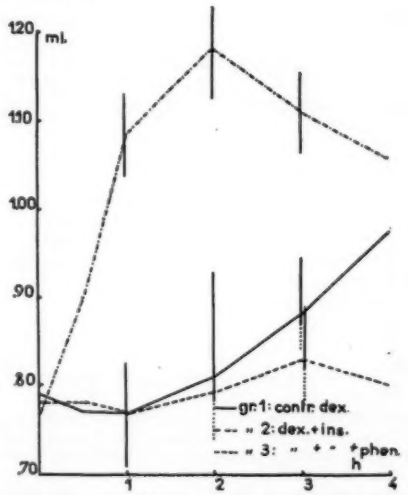

FIG. 1. Inhibition by phenergan of insulin sensitization to "anaphylactoid" inflammation. Abscissa: hours after injection of dextran (irritant). Ordinate: volume in ml. of left hind paw (general "anaphylactoid" inflammation). Group I, rats injected with dextran s.c. on back: swelling of paw is small and occurs late. Group II, rats similarly injected with dextran, but which also received insulin: swelling now rapid and intense. Group III, rats treated as in Group II, plus phenergan: paw does not swell, the sensitization effect of insulin is abolished (vertical lines represent standard error [S.E.] ×2).

II. Desensitization of Rats to "Anaphylactoid" Inflammation by Means of Compound 48:80

Thirty-eight rats were treated with 48:80. Initially, their mean body weight was 98 g. It reached 180 g. at the end of the 25 days of treatment. Growth was therefore normal. The injections of 48:80 produced an "anaphylactoid" inflammation in all the rats, every day, until the 20th day. After this, the number of rats becoming inflamed decreased rapidly. On the 23rd day, none of the rats reacted following the daily injection of 48:80. The injections were continued until the 25th day. On the 26th day, the rats were used for experiment III.

III. Inhibition, by the Histamine Depletor, of the Insulin Sensitization to "Anaphylactoid" Inflammation

Eighteen desensitized rats from experiment II, weighing 160–180 g., were divided into two groups of nine. Group I received 1 ml. dextran i.p., Group II received 1 ml. dextran s.c. on the back plus four units of insulin.

None of the rats showed inflammation, Fig. 2.

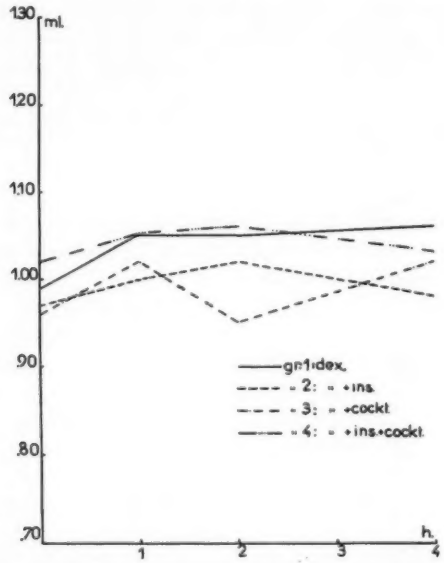

FIG. 2. Inhibition by 48:80 of insulin sensitization to "anaphylactoid" inflammation. All rats are pretreated 25 days with 48:80. Group I, there is no swelling although dextran is injected i.p. Group II, no swelling, although rats, which are injected with dextran s.c., are sensitized with insulin. Group III, no swelling after i.p. injection of dextran, although rats received simultaneous injections of histamine–serotonin–heparin mixture. Group IV, no swelling although rats which were injected s.c. with dextran received the mixture and were sensitized with insulin.

IV. Persistence of Inhibition of the "Anaphylactoid" Inflammation in Desensitized Rats Treated with a Histamine–Serotonin–Heparin Mixture

A further 18 desensitized rats from Experiment II weighing 165–180 g., were divided into two groups of nine: Groups III and IV, Fig. 2. Group III received 1 ml. dextran i.p. plus one i.p. injection of the mixture containing 1 mg. histamine, 1 mg. heparin, and 100 μg. serotonin. This mixture constituted a partial substitution for the mast cells destroyed with the 48:80. Group IV received a similar injection of the mixture, an injection of 1 ml. dextran s.c. on the back, and a sensitizing dose of four units insulin s.c.

None of the animals became inflamed.

In another experiment, not fully reported here, the mixture was injected i.p. every half hour, for several hours, into desensitized rats, which had been injected i.p. with dextran. Again none of the animals reacted with "anaphylactoid" inflammation.

V. Histamine, Serotonin, and Heparin do not Sensitize to "Anaphylactoid" Inflammation

Six groups of six rats each were treated as follows:

Group I, 0.5 ml. dextran s.c. on the back.

Group II, dextran *idem*, plus four units insulin, s.c.

Group III, dextran *idem*, plus 1 mg. histamine, i.p.

Group IV, dextran *idem*, plus 1 mg. heparin, i.p.

Group V, dextran *idem*, plus 100 μg. serotonin, i.p.

Group VI, dextran *idem*, plus mixture containing above quantities of histamine, heparin, and serotonin, i.p.

As seen in Fig. 3, the only group of rats which became inflamed was Group II, where insulin was administered.

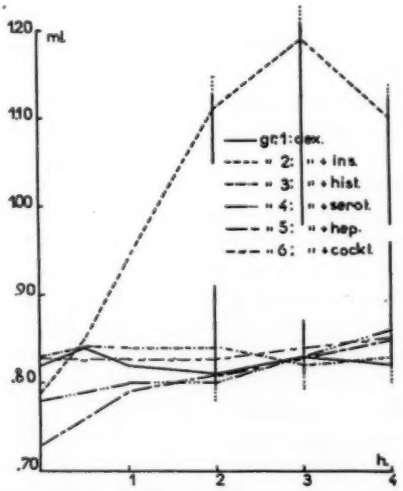

FIG. 3. Histamine, serotonin, and heparin do not sensitize to "anaphylactoid" inflammation. All rats received a s.c. injection of dextran on the back. In addition Group II received insulin; Group III, histamine; Group IV, serotonin; Group V, heparin; and Group VI, the histamine–serotonin–heparin mixture. Only rats of Group II, receiving insulin, became inflamed (vertical lines represent S.E. ×2).

VI. Effect of Insulin on Capillary Permeability

Twenty-four hours before the beginning of this experiment, the abdomens of 40 rats, weighing 120–150 g., were shaved. The following day, the animals were ether anaesthetized, one by one, and were injected with Evans blue i.v. On the shaven abdomens were placed, as widely apart as possible, four s.c. injections, each consisting of 0.1 ml. of a saline solution containing the following substances:

Spot 1: only saline	Spot 3: 5 μg. dextran
Spot 2: 5 μg. histamine	Spot 4: 5 μg. insulin

The spots on each rat were inspected every 15 minutes. The blue coloration which appeared there was assessed by giving it 0 to 3 marks, according to its intensity (no color 0; traces of color 1; well marked coloration 2; very well marked coloration 3). After 1 hour of observation averages were calculated for each substance tested.

The results shown in Fig. 4 indicate that the highest degree of leakage of Evans blue from capillaries was induced by histamine: 2.24 ± 0.09 marks per rat. Dextran induced a leakage of 1.58 ± 0.05 marks per rat. The leakages induced by saline, 0.33 ± 0.09 marks per rat, and by insulin, 0.05 ± 0.02 marks per rat, were negligible. The differences between insulin and histamine, and between insulin and dextran were highly significant statistically: $p < 0.01$.

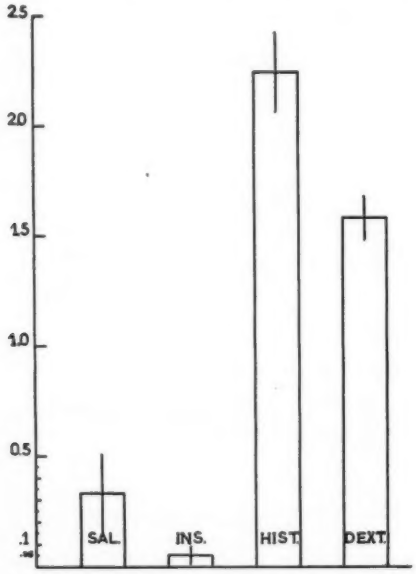

FIG. 4. Effect of insulin on capillary permeability in the rat. Ordinate: index of intensity of capillary permeability; possible maximum 3 (see experiment VI). Histamine induces a nearly maximal response of capillary permeability. Insulin induced response is negligible (vertical lines represent S.E. ×2).

Discussion

In an attempt to elucidate the mechanism of insulin sensitization to "anaphylactoid" inflammation, we tested the hypothesis that the sensitizing effect of insulin is mediated through histamine. Insulin would liberate histamine, directly or indirectly. The extra histamine would be responsible for increased sensitivity of rats to "anaphylactoid" agents.

The first two experiments seemed to support such a view. Rats treated with phenergan, an antihistaminic (experiment I), ceased to react to the "anaphylactoid" agent, and could not be sensitized to it by insulin. In presence of an antihistaminic, the sensitizing property of insulin vanished. Prolonged treatment with compound 48:80, a histamine depletor (10), also abolished the "anaphylactoid" inflammation, and the sensitizing property of insulin (experiment III). The lowering of tissue histamine levels by a histamine depletor again caused the sensitizing effect of insulin to vanish.

A prolonged treatment with compound 48:80 induces a considerable destruction of mast cells (9), which, in the rat, are important sites of histamine, serotonin, and heparin. This destruction coincides with the disappearance of "anaphylactoid" reactivity. It could be supposed therefore that the disappearance of reactivity is linked with the decrease in the amount of some components of mast cells. If so, a partial substitution of constituents of mast cells could lead to a reappearance of "anaphylactoid" reactivity. The 48:80 pretreated rats were injected, therefore, systemically with a mixture consisting of histamine, serotonin, and heparin. However, the "anaphylactoid" reactivity and the insulin sensitization to it (experiment IV), did not reappear.

Experiment V furthermore illustrates that the three components of mast cells, administered systemically, do not sensitize the normal rat to "anaphylactoid" reactions as insulin does.

However, during "anaphylactoid" reactions, the components of mast cells could be liberated locally, in large amounts, in those tissues where the reaction takes place (snout, ears, paws, and the genital and anal regions). A systemic administration of the mast cell components does not reproduce such events.

An indirect evidence on a possible local liberation of mast cell components by insulin was obtained from experiment VI. During the course of this experiment, the topical administration of insulin did not induce local discharge of histamine, as judged by the leakage of Evans blue from capillaries at the site of insulin injection. This leakage was negligible when compared to that induced by histamine itself or by dextran, which is a known histamine liberator.

In conclusion, we suggest that the sensitizing effect of insulin on "anaphylactoid" inflammation could be merely an incidental manifestation of a more fundamental property of the hormone. For example, the insulin could accelerate the access of dextran to those mechanisms which undergo the "anaphylactoid" reaction. The acceleration may be independent of the components of mast cells. But, if the "anaphylactoid" reaction itself is inhibited by phenergan or 48:80, the accelerating action of insulin cannot be detected with the present experimental arrangement, as was the case in experiments I and II.

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METHYLATION OF THE 3-OH POSITION OF CATECHOL ACIDS BY RAT LIVER AND KIDNEY PREPARATIONS[1]

J. PELLERIN AND A. D'IORIO

Abstract

3,4-Dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, and 3,4-dihydroxycinnamic acid were separately incubated with L-methionine-methyl-C^{14} in the presence of rat liver or kidney homogenate. In each case, the radioactive metabolite separated by paper chromatography was found to have migrating properties similar to those of the 3-methoxy-4-hydroxyphenolic acid. This reaction was enhanced by the addition of ATP, Mg^{++}, and reduced glutathione. When 3-hydroxybenzoic acid was incubated in this medium no methylated derivative was obtained. Preliminary experiments indicated that the enzymatic activity was contained mostly in the supernatant fraction. It was also noted that liver homogenate was much more active than kidney homogenate in methylating catechol acids.

In 1951, Maclagan and Wilkinson (8) reported that injection of 4-hydroxy-3,5-diiodobutylbenzoate to human subjects resulted in the urinary excretion of 4-methoxy-3,5-diiodobenzoic acid. More recently, several authors (12, 4, 9) have demonstrated the urinary excretion of 3-methoxy-4-hydroxyphenolic acids.

The present work is concerned with the *in vitro* study of O-methylation of catechol acids.

Experimental

Chemicals

Radioactive L-methionine-methyl-C^{14} was purchased from Isotopes Specialties and had a specific activity of 1.2×10^6 c.p.m./mg.

Samples of 3,4-dihydroxymandelic acid (V), 3-methoxy-4-hydroxymandelic acid (VI), and homovanillic acid (IV) were kindly supplied to us by Drs. M. D. Armstrong and K. N. F. Shaw of the University of Utah. Dr. C. W. Murray of the Agricultural Research Service, Albany, Calif., generously provided pure samples of 3,4-dihydroxyphenylacetic acid (III). The other phenolic acids and their methoxy derivatives, protocatechuic acid (I), vanillic acid (II), caffeic acid (VII), ferulic acid (VIII), 3-hydroxybenzoic acid (IX), and 3-methoxybenzoic acid (X) were purchased from K and K Laboratories, Long Island City, N.Y.

Incubation

Five substrates have been tested for their ability to undergo O-methylation namely: 3,4-dihydroxybenzoic acid (I), 3,4-dihydroxyphenylacetic acid (III), 3,4-dihydroxycinnamic acid (VII), 3,4-dihydroxymandelic acid (IV), and 3-hydroxybenzoic acid (IX). The structural formulae of the substrates and of their methylated derivatives are listed in Fig. 1.

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Contribution from Laboratoire de Physiologie, Université de Montréal, Montreal, Que.

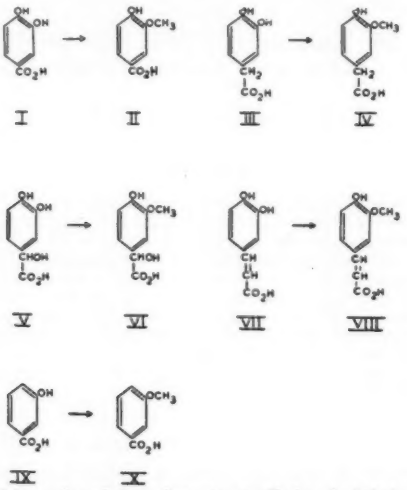

FIG. 1. Structural formulae of the substrates studied and of their 3-methoxy derivatives.
I—3,4-Dihydroxybenzoic acid (protocatechuic acid).
II—3-Methoxy-4-hydroxybenzoic acid (vanillic acid).
III—3,4-Dihydroxyphenylacetic acid.
IV—3-Methoxy-4-hydroxyphenylacetic acid (homovanillic acid).
V—3,4-Dihydroxymandelic acid.
VI—3-Methoxy-4-hydroxymandelic acid.
VII—3,4-Dihydroxycinnamic acid (caffeic acid).
VIII—3-Methoxy-4-hydroxycinnamic acid (ferulic acid).
IX—3-Hydroxybenzoic acid.
X—3-Methoxybenzoic acid.

The phenolic acids were incubated in a Warburg vessel containing: 2 ml. of rat liver or kidney homogenate (50% w/v), 250 μg. of the neutralized phenolic acids, 38 μM. of phosphate buffer (pH 7.4), 5.8 μM. of L-methionine-methyl-C^{14} (1.2×10^6 c.p.m./mg.), 7.3 μM. of ATP (neutralized to pH 7.4), 500 μM. of $MgCl_2$, 9.6 μM. of reduced glutathione, and, in the center well, 6 μM. of 2 *N* NaOH. The reaction medium had a total volume of 3.35 ml. and was incubated at 37° C. for 2 hours.

In some experiments the total homogenate was replaced by the following fractions: (*a*) supernatant, (*b*) mitochondria plus microsomes. The separation of these fractions was achieved in isotonic sucrose using the procedure described by Schneider (11). Following incubation the reaction was stopped by the addition of 0.2 ml. of concentrated hydrochloric acid. The protein was removed by centrifugation and sodium chloride was added to the supernatant to a final concentration of 10%. The pH of the supernatant was adjusted to 1 and the clear solution was extracted three times with equal volumes of ethyl acetate. This extraction procedure was tested with all of the pure substrates and methoxy derivatives previously mentioned with amounts and volumes equivalent to those obtained with incubation medium. Quantitative estimation of these compounds in the trial experiments was achieved by measuring the U.V. absorption at 279 mμ. The recovery of these substances always exceeded 95%.

In the experiments where catechol acids were incubated with L-methionine-methyl-C^{14} the methoxy derivatives present in the ethyl acetate extracts were separated and identified by paper chromatography. The general procedure was similar to that previously described (10). The ethyl acetate extracts were concentrated *in vacuo* to a small volume. These concentrates were then chromatographed on Whatman No. 1 paper, using the descending technique. All chromatograms were routinely developed in three solvent systems. Solvent A, consisted of benzene: propionic acid: water, 2:2:1 (2); solvent B, *n*-butanol: 1 *N* hydrochloric acid, 4:1; and solvent C, chloroform: glacial acetic acid: water, 2:1:1(6). Generally two identical spots were applied on the starting line several centimeters apart. These two spots contained an aliquot of the ethyl acetate extract plus 5 μg. of pure methoxy derivative.

Upon completion of the chromatographic separation, the papers were dried at room temperature and cut vertically in two parts. The first half was sprayed with the potassium ferricyanide – ferric sulphate reagent of Goldenberg *et al.* (7) to localize the position of the control substances. The second half was cut horizontally in 1 cm. strips. These strips were eluted with 0.01 *N* HCl. The eluates were collected in cupped planchets and evaporated to dryness and their radioactivity was measured in a gas flow counter.

Results

R_f Values of Control Substances

The pure phenolic acids and their methoxy analogues were chromatographed in the three solvent systems described. These compounds were spotted from ethyl acetate solution and their R_f values are reported in Table I.

TABLE I

R_f VALUES OF CONTROL SUBSTANCES

Compounds	Solvent A, benzene: propionic acid: water (2:2:1) (3 hr.)	Solvent B, BuOH: 1 *N* HCl (4:1) (16 hr.)	Solvent C, $CHCl_3$: glacial acetic acid: water (2:1:1) (4 hr.)
Protocatechuic acid	0.20	0.73	0.16
Vanillic acid	0.70	0.79	0.72
3,4-Dihydroxyphenylacetic acid	0.20	0.69	0.19
Homovanillic acid	0.65	0.76	0.72
Caffeic acid	0.24	0.73	0.23
Ferulic acid	0.70	0.79	0.74
3,4-Dihydroxymandelic acid	0.12	0.59	0.08
3-Methoxy-4-hydroxymandelic acid	0.22	0.62	0.26
3-Hydroxybenzoic acid	0.52	—	—

As can be seen in this table no R_f value is given for 3-methoxybenzoic acid. This is explained by the fact that since this compound has no free hydroxyl group, no coloration was obtained with the ferricyanide – ferric sulphate reagent.

Radioactivity of Methoxyphenolic Acids Following Incubation

The results summarized in Table II are representative of four such experiments. The incubation of the various substrates was conducted simultaneously in a parallel fashion using aliquots of the same kidney or liver homogenate.

TABLE II

O-METHYLATION OF 3,4-DIHYDROXYPHENOLIC ACIDS BY RAT LIVER AND KIDNEY HOMOGENATES

Substrates	Incubation medium	3-Methoxyphenolic acid formed	Total radioactivity of 3-methoxyphenolic acids,* counts per minute per g. of fresh tissue	
			Liver homogenate	Kidney homogenate
Protocatechuic acid	Complete	Vanillic acid	82492	1790
	Without cofactors†	Vanillic acid	16838	2075
3,4-Dihydroxyphenylacetic acid	Complete	Homovanillic acid	79564	1722
	Without cofactors	Homovanillic acid	15800	1545
Caffeic acid	Complete	Ferulic acid	40480	1408
	Without cofactors	Ferulic acid	5425	704
3,4-Dihydroxymandelic acid	Complete	3-Methoxy-4-hydroxymandelic acid	8357	—
	Without cofactors		5915	—
3-Hydroxybenzoic acid	Complete	—	Nil	Nil
	Without cofactors	—	Nil	Nil

*Radioactivity of L-methionine incubated 9.4×10^6 c.p.m.
†Cofactors 7.3 μM. of ATP, 500 μM. of $MgCl_2$, and 9.6 μM. of glutathione.

The first three compounds listed: vanillic acid (II), homovanillic acid (IV), and ferulic acid (VIII) represented in each case over 90% of the total radioactivity found in the ethyl acetate fraction. In some experiments a secondary peak representing 5 to 10% of the total activity could be found. The nature of this secondary peak is unknown, but it is certainly related to the presence of the substrate since no significant radioactivity was found in ethyl acetate extracts of endogenous blanks where radioactive methionine was incubated in the presence of tissues without phenolic acids. When incubating 3-hydroxybenzoic acid (IX), little or no radioactivity was found in the ethyl acetate extract.

The presence of magnesium chloride, adenosine triphosphate, and reduced glutathione greatly enhanced the formation of the 3-methoxy derivatives of protocatechuic acid (I), 3,4-dihydroxyphenylacetic acid (III), and of caffeic acid (VII). When using 3,4-dihydroxymandelic acid (V) as substrate, the previously mentioned cofactors exerted very little influence.

However, with this latter substrate a secondary reaction occurred and only 40% of the total activity of the ethyl acetate extract had migrating properties identical with 3-methoxy-4-hydroxymandelic acid (VI). The distribution of the radioactive components following incubation of 3,4-dihydroxymandelic acid (V) and radioactive methionine in the presence of liver homogenate is shown in Fig. 2.

The R_f of 3-methoxy-4-hydroxymandelic acid (VI) was identical in all three solvents with that of peak 1 while the R_f of peak 2 in the three solvent systems was identical with that of vanillic acid (II).

To establish further that the observed radioactivity was located in the carbon of the methoxy group the following procedure was applied. The radioactive ferulic acid (VIII) isolated by paper chromatography was eluted and 10 mg. of carrier ferulic acid was added. This compound was demethylated by the modified Pregl procedure (5). The methyl iodide formed was bubbled through triethylamine thus leading to the formation of methyl-triethylammonium iodide. The radioactivity of the latter compound was found to be 95% of the initial radioactivity of the ferulic acid (VIII).

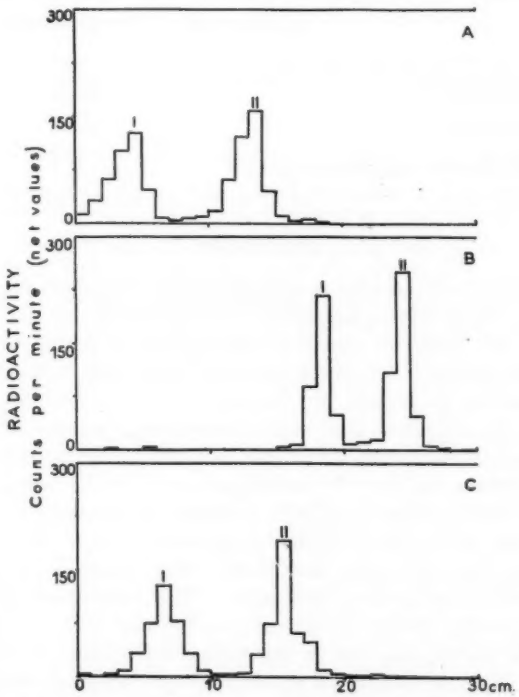

FIG. 2. Paper chromatography of ethyl acetate extracts obtained following incubation of 3,4-dihydroxymandelic acid with rat liver homogenate.
A. Solvent system: benzene: propionic acid: water (2:2:1), 3 hours.
B. Solvent system: butanol: 1 *N* hydrochloric acid (4:1), 16 hours.
C. Solvent system: chloroform: glacial acetic acid: water (2:1:1), 4 hours.

Intracellular Distribution of the Enzymatic Activity

In three experiments using rat liver homogenates, supernatant and mitochondria plus microsomes were prepared and incubated with protocatechuic acid (I) and radioactive methionine. In each experiment duplicate runs were

carried out. The quantity of supernatant and of mitochondria plus microsomes in the incubation mixture was equivalent to that found in 1 g. of rat liver. The radioactivity of the vanillic acid (II) eluted from the paper chromatograms was measured. The activity observed with the total homogenate was taken as 100 and the activity of the other fractions expressed accordingly. The results of one such experiment are shown in Table III and indicate that the activity of the supernatant is greater than that of the total homogenate.

TABLE III

INTRACELLULAR DISTRIBUTION OF THE METHYLATING ENZYME IN RAT LIVER HOMOGENATES

	Relative activity in %
Homogenate	100.0
Supernatant	234.0
Mitochondria and microsomes	2.1

Discussion

On the basis of the experiments reported in the present work, it can be seen that the liver and kidney homogenates can methylate the 3-hydroxyl group of catechol acids. However, the liver homogenate is much more active than the kidney homogenate. The methylation of these acids by total homogenates seems to depend on the length and complexity of the side chain. The results reported in Table I indicate that with a one-carbon or two-carbon side chain there is an equivalent formation of the 3-methoxy compounds. When the side chain is composed of three carbon atoms and a double bond, as in the case of caffeic acid (VII), there is a 50% decrease of the activity. The lowest values were obtained with 3,4-dihydroxymandelic acid (V). However, in this case a secondary metabolite was found which accounts for nearly 60% of the radioactivity on the chromatograms. This radioactive metabolite had migrating properties similar to that of vanillic acid (II). It could then be proposed that, either prior to or following methylation of 3,4-dihydroxymandelic acid (V), this compound would undergo oxidation and decarboxylation. Furthermore, since the major urinary metabolites of adrenaline and noradrenaline have been found to be 3,4-dihydroxymandelic acid (V) and its 3-methoxy derivatives VI (1), it is logical to believe that the vanillic acid (II) normally present in urine (2) also derives from adrenaline and noradrenaline.

When 3-hydroxybenzoic acid (IX) was used as substrate, no radioactivity could be located on the paper chromatograms. This would indicate that the hydroxyl in position 4 is essential to the methylating enzyme system.

The present work further indicates that the methylating enzymes are located in the supernatant fraction and that mitochondria and microsomes exert an

inhibitory action. This inhibition is probably due to the ATPase activity of these fractions, which would destroy the ATP necessary to methylation.

Recently, Axelrod (3) has reported a similar methylation for catechol amines using a purified liver enzyme. He has further shown that S-adenosylmethionine can act as a methyl donor for this reaction. A survey of the biological activity of the methoxy derivatives of catechol acids and catechol amines would be of great interest because of their structural analogy with the natural catechol amines and with psychogenic substances such as mescaline.

Acknowledgment

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THE SURVIVAL OF MAMMALIAN TISSUES PERFUSED WITH INTRAVASCULAR GAS MIXTURES OF OXYGEN AND CARBON DIOXIDE[1]

B. DELISLE BURNS, J. G. ROBSON, AND G. K. SMITH

Abstract

It is possible to maintain active mammalian cardiac and skeletal muscle in good condition for 3 or 4 hours by the intravascular perfusion of these tissues with warm, moist oxygen containing 5% carbon dioxide. Some of the uses of this perfusion technique are discussed.

Introduction

In 1954 Bunzl *et al.* (3) described experiments in which the spinal cords of decerebrate frogs were maintained in a reflexly active state for 24 hours when the vascular system was perfused with a gas mixture of oxygen and 3% carbon dioxide. These workers found perfusion of the frog's vascular system with saline solutions to be inconvenient for a number of reasons. Perfusion with oxygenated saline solutions led to the rapid formation of oedema; when proteins were added to the perfusion medium, the viscosity became so great that adequate oxygenation of the spinal cord became impossible. Intravascular perfusion of the experimental animals with moist gas mixtures was therefore adopted by Bunzl *et al.*, since this technique (1) allowed the maintenance of normal reflex activity of the spinal cord for many hours, (2) produced no oedema, (3) permitted the administration of drugs, in short columns of fluid, and (4) made possible the collection of venous effluents in which metabolic products could be collected, dissolved in very small quantities of water.

There appeared to be no obvious reason why mammalian tissues should not survive such treatment. Because this technique of perfusion might be a useful tool in the hands of the pharmacologist and physiologist, we decided to investigate the survival of some active mammalian tissues perfused intravascularly with various gas mixtures. This paper presents some preliminary work on the survival of the isolated rabbit's heart during perfusion with intravascular oxygen, and also the survival under similar conditions of the cat's active tibialis anterior muscle.

Methods

Since the experiments we shall describe are solely concerned with the exploration of an experimental method, only the part of our technique that was common to all experiments will be described below.

The gas mixture used for perfusion was led from a cylinder and reducing valve to a sintered glass bubbler in a reservoir containing 0.9% NaCl solution, maintained at 37° C. The moist gas then passed through a glass warming

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Contribution from the Department of Physiology and the Wellcome Research Department of Anaesthesia, McGill University, Montreal, Que.

coil in a water bath at 37° C. to ensure that it reached the perfused organ at body temperature. A Bourdon-type pressure gauge was connected to the gas supply line, close to the arterial cannula, for registration of the perfusion pressure. Various taps and branches were included in the gas supply line to make possible such manoeuvres as the injection of small columns of saline into the perfusate.

During those experiments in which the flow of the gas perfusate was measured, a glass capillary was placed in the supply line. The pressure head across this capillary was measured with Bourdon gauges; flow through this constriction was calibrated in terms of the pressure drop across the capillary.

When an organ was connected for perfusion with gas, a cannula was first tied into the appropriate artery and the blood was swept out of the organ with warm, oxygenated Tyrode's solution containing 0.2% glucose. Gas was then permitted to displace saline from the vascular system at a pressure of about 120 mm. Hg. Once the gas had made its way into the venous system the supply pressure was reduced to about 80 mm. Hg.

Results

The Survival of Active Cardiac Muscle

A rabbit weighing about four kilograms was stunned by a blow on the head, the thorax was opened, and finally the heart together with stumps of the great vessels was removed as rapidly as possible. A cannula was tied into the aorta with its tip pointing toward the aortic valves, care being taken that the cannula should not interfere with the proper closure of these valves. Perfusion with Tyrode's solution was begun immediately; the base and apex of the heart were then fixed to glass rods with threads sewn through the myocardium and a thread passed through the wall of the left ventricle served to operate an isotonic lever which indicated contractions of the heart on a smoked drum. Finally, the arterial cannula was connected to the supply of moist oxygen and 5% carbon dioxide. To ensure that the only source of oxygen for the preparation should be through the coronary vessels, the heart was suspended throughout the experiment in an atmosphere of warm, moist nitrogen. Occasionally, a slow leakage of gas back through the aortic valves made it necessary to slit the left ventricular wall in order to avoid excessive distention of the heart.

Under these conditions of perfusion, the heart maintained a regular beat, the amplitude of which diminished little (less than 20%) during a period of more than three hours. That the survival of the normal beat did in fact depend upon the continued intravascular gas supply was demonstrated by switching the perfusion, temporarily, to nitrogen. Fig. 1 shows a record of the effect of warm, moist, intravascular nitrogen upon the amplitude of the heart beat. Three minutes after the switch to nitrogen there was a temporary augmentation of the beat, following which the contractions declined precipitously until the oxygen supply was restored to the circulation.

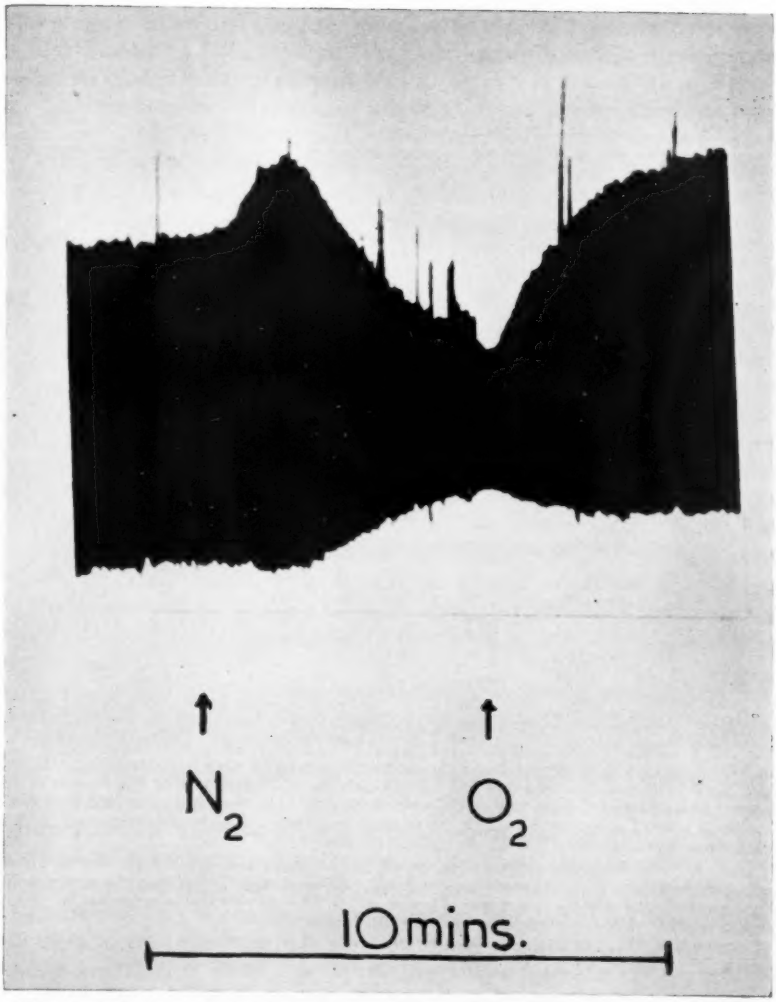

FIG. 1. Isotonic record of contractions of the rabbit's cardiac ventricle during gas perfusion of the coronary vessels. Moist nitrogen was exchanged for the oxygen–CO_2 mixture (used in the experiment of Fig. 1).

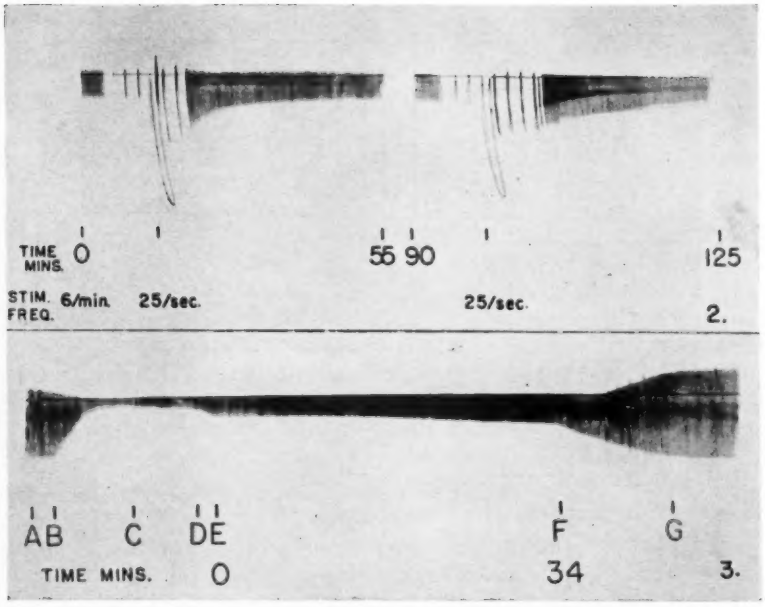

FIG. 2. Isometric contractions of the cat's tibialis anterior muscle, during intravascular perfusion with moist oxygen and 5% carbon dioxide. (Contraction of the muscle is recorded as a downward deflection of the writing lever.) The muscle was excited by supramaximal stimuli given to the sciatic nerve at 1 in 10 sec. The tetanic responses to two periods of repeated stimuli at 25 per second are shown.

FIG. 3. The isometric contractions of the cat's tibialis anterior muscle during intravascular perfusion with moist oxygen and 5% carbon dioxide. The muscle was excited by supramaximal stimuli to the sciatic nerve at 1 in 10 sec. Partial neuromuscular block was produced with *d*-tubocurarine as described in the text.

After a period of 3–4 hours of perfusion with oxygen, the beat of most of the hearts we tested began to weaken. We found, however, that they could be revived by admitting oxygenated Tyrode's solution (with 0.2% glucose) for about two minutes and then returning to the supply of intravascular oxygen. By treatment of this sort we have occasionally been able to maintain hearts on an intravascular gas supply for as long as 9 hours.

The gas flow required to maintain a rabbit's heart in good condition was of the order of 20 ml./g./minute. This flow of oxygen is of course much larger than that needed for metabolism; Fig. 4 demonstrates that this flow is necessary only to maintain the vessels open.

The Survival of Active Skeletal Muscle

Similar experiments demonstrated that intravascular perfusion of the cat's hind limb with moist, warm oxygen containing 5% carbon dioxide will maintain an exercised tibialis anterior muscle for 3 to 4 hours.

The cats used for these experiments were anaesthetized with chloralose; the femoral artery was exposed for cannulation and stimulating electrodes were placed upon the sciatic nerve in the thigh. The sciatic nerve was tied central to the stimulating electrodes and the branch supplying the gastrocnemius and soleus muscles was severed. A stout ligature was used as a tourniquet around the upper leg to prevent any collateral circulation to the lower leg from the body of the cat. This ligature was, of course, applied so as to exclude the femoral artery, the femoral vein, and the sciatic nerve. The distal tendon of the tibialis anterior was freed from its bony insertion and connected to an isometric writing lever recording on a smoked drum; clamped bone drills inserted into the tibia and femur were used to steady the leg during the experiment.

When the preparation had been arranged for recording the contractions of tibialis, the femoral artery was cannulated and the femoral vein was slit. Blood was swept from the vascular system with warm oxygenated Tyrode's solution (+0.2% glucose) and the arterial cannula was then connected to the oxygen supply.

Perfused in this way, the tibialis muscle which was excited to contract once every 10 seconds by supramaximal, single stimuli of the sciatic nerve, maintained an undiminished twitch tension for 3 or 4 hours. Fig. 2 shows the responses of such a muscle at the beginning and end of a 2-hour period; it will be seen that the muscle is still capable of a good tetanic response to repetitive stimulation after 2 hours of oxygen perfusion. The records of Fig. 2 show also the well-known posttetanic potentiation (2).

The Maintenance of a Constant Degree of Neuromuscular Block

In the study of neuromuscular facilitation it has frequently proved desirable to maintain a constant degree of neuromuscular block for relatively long periods of time, in order to provide a stable background of subnormal neuromuscular conduction against which the facilitating action of various manoeuvres and drugs may be studied (see for instance, references 1 and 4). The

maintenance of an unvarying degree of neuromuscular block is usually effected by the slow intravenous infusion of an agent such as *d*-tubocurarine; it is, however, very difficult to adjust the rate of infusion of blocking agent so that the degree of block is stable.

Intravascular oxygen perfusion considerably simplifies the problem of maintaining a constant level of drug activity where the drug is not destroyed at its site of action. The objective of the experiment, results of which are illustrated in Fig. 3, was to achieve approximately 50% neuromuscular block and then to maintain this degree of block unchanged for many minutes. The test muscle was perfused with the usual gas mixture of moist oxygen with 5% carbon dioxide. The responses of the muscle to supramaximal stimuli given to the sciatic nerve once every 10 seconds are shown. Between A and B, 10^{-4} *d*-tubocurarine in 10 cc. of Tyrode's solution was admitted to the arterial cannula and was swept through the preparation by the gas. This treatment produced too great a degree of neuromuscular block; consequently at C and D two short columns of Tyrode's solution were passed through the preparation to sweep out the excess curare. At E, it was judged that the degree of block was satisfactory and the preparation was left on oxygen perfusion for 34 minutes until F. Between F and G, oxygenated Tyrode's solution was used to restore neuromuscular conduction to normal. At G the preparation was again supplied with intravascular oxygen.

The Conditions Necessary for Satisfactory Perfusion

Early in this series of experiments we noticed that a much higher pressure of gas was necessary to force fluid from the vascular bed of the isolated organ than was required subsequently to maintain a satisfactory flow of oxygen.

Measurements of the relations between supply pressure and intravascular flow show that the curve relating the two demonstrates hysteresis. Fig. 4 illustrates the results obtained relating pressure to flow of gas through the isolated rabbit's heart. When the coronary vessels were filled with saline solution the supply pressure had to be raised to 60 mm. Hg before any flow took place; further increase of gas pressure produced a corresponding increase of

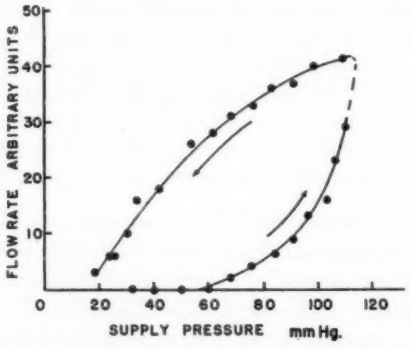

FIG. 4. The relation between supply pressure and flow of a gas mixture (moist O_2+5% CO_2) through the coronary vessels of the active rabbit's heart.

flow, and there was a dramatic increase of flow when the supply pressure reached about 110 mm. Hg. If the arterial gas pressure was then reduced it was found that considerable flow through the vascular tree could be maintained at pressures which were incapable of initiating gas flow. These curves proved to be repeatable provided that the starting pressure was zero. Similar results were obtained for perfusion of the cat's hind limb.

For any individual small vessel within the heart, the sweeping out of fluid by gas must provide an all-or-nothing relation between supply pressure and flow through this vessel. The comparatively slow rise of flow with increase of pressure (beyond the threshold opening pressure) observed in the whole heart is presumably due to the presence of many different diameters of small vessel, scattered about a mean value.

Discussion

The hysteresis of the pressure flow curves, described in the last section of this report, is largely due to the fact that greater pressures must be used to overcome the surface tension of fluid in the smaller vessels than is necessary to maintain a flow through these vessels once a channel for gas has been established. The simplest relation between the pressure (P) needed to drive gas through a tube (of radius r) containing liquid, and the surface tension (T) of the gas–liquid interface is:

$$P = 2T/r$$

where T is in dynes/cm.; P is in dynes/cm.^{2}; and r is in cm.

The surface tension at our air–water interface is about 70 dynes/cm. (6). The saline solution, which we were trying to drive from the vascular bed, was presumably mixed with some plasma; the surface tension of human serum is about 50 dynes/cm. (8). We have therefore assumed that about 60 dynes/cm. was resisting the entry of gas to the vascular bed. Using this value for T, and assuming an average capillary diameter of 5μ (5), $P = 360$ mm. Hg. This is much greater than the observed value for P, but there are at least two reasons for this discrepancy:

(1) At the moment when gas is just about to force its way into a capillary, the vessel will be subjected to the full supply pressure (approximately normal systolic arterial pressure). This will mean that in the critical condition capillaries will be passively dilated.

(2) The formula that is used above assumes perfect wetting of the vessel walls by the fluid, and consequently that the contact angle of interface and vessel wall was zero. There is evidence that the walls of healthy vessels are not wettable; soon after death the properties of vascular endothelium change so that after 45 minutes it is fully wettable (9). Where the contact angle (α) is not zero, the formula relating P and T becomes

$$P = 2T \cos \alpha / r.$$

If we were to assume a value for mean capillary diameter of 10μ and a contact angle (α) of 45°, then

$$P = 128 \text{ mm. Hg.}$$

We were surprised to find that active mammalian muscle could survive for so long without change in its capacity for work under conditions where there can be no removal of the metabolites of activity. This observation makes it seem very unlikely that the cardiac arrhythmia seen sometimes during operations under hypothermia can be due to the accumulation of extracellular potassium ions (10) arising from the heart itself. Nevertheless, the results of the experiments described above do not add much to our understanding of the functions of the organs which we have perfused. They do little more than illustrate a new method for the maintenance of isolated organs. We feel that this method may prove to be a useful technique. Theoretically it should:

(1) allow prolonged perfusion of tissues without oedema formation;

(2) facilitate the maintenance of constant effects from drugs which are not destroyed at their site of action, but which are rapidly excreted or destroyed elsewhere in the circulation;

(3) facilitate the collection of metabolites, humoral transmitters, etc., in relatively concentrated solutions.

We do not yet know whether gas perfusion would play a useful role in human surgery; nor do we know what organs in the body other than muscles would survive similar treatment. Although the spinal cord of the frog will survive intravascular oxygen perfusion for many hours (3), we have not succeeded in maintaining the mammalian central nervous system in this manner. In five anaesthetized cats perfusion of the whole animal with intravascular oxygen led to an immediate end of respiration. This may be because the metabolic rate of the central nervous system is comparatively great and its stores of glycogen are small (7).

Acknowledgments

We would like to express our thanks to Dr. A. S. V. Burgen, who assisted with some of the early experiments and with whom we have had much helpful discussion. We are indebted also to the National Research Council of Canada, which contributed support to this work through a grant to one of us.

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HYPERGLYCEMIC ACTION AND TOXICITY OF THE URINE OF SCHIZOPHRENIC PATIENTS[1]

F. Moya, J. Dewar, M. MacIntosh, S. Hirsch, and R. Townsend

Abstract

The findings presented confirm earlier reports in the literature that extracts prepared from the urine of schizophrenic patients have greater hyperglycemic activity than similar preparations from normal subjects. In addition, it has been found that the patients' extracts are quite toxic to the rabbit whereas preparations from normal urine are not.

Preliminary studies indicate that extracts from the urine of mental patients other than acute schizophrenics behave like extracts from the urine of normal subjects.

Introduction

In 1948 Meduna and Vaichulis (1) reported that extracts prepared with the aid of kaolin from the urine of either normal subjects or schizophrenic patients were hyperglycemic when injected intraperitoneally into rabbits. Experiments in this laboratory have shown that extracts prepared by treating normal urine with ethanol are hyperglycemic (3) and this action is due to the presence of the hypotensive agent kallikrein (4). It has also been found that the hyperglycemic response to the intravenous administration of extracts of normal urine prepared by the procedure of Meduna *et al.* can be inhibited by adrenolytic agents (3).

The present communication deals with a reinvestigation of the hyperglycemic activity of extracts prepared from the urine of normal and schizophrenic women by a procedure similar to the one described by Meduna *et al.* In addition the toxicity of these two types of extracts has been studied.

Materials and Methods

The normal donors were healthy, female laboratory workers. The patients used in this study were schizophrenic women. They were all actively ill with delusions, hallucinations, and thinking disorders. Only those cases were chosen in which there was no doubt of the diagnosis. At least two psychiatrists evaluated every patient. The ages ranged from 18 to 43 years. The duration of illness varied from several months to many years. In all cases, hospitalization in a mental hospital was necessitated by an acute phase of the psychosis. The specimens of urine were collected within several days of admission, at a time when the patients were not under treatment.

The non-schizophrenic patients studied comprised six females ranging in age from 24 to 67 years. At the time the urine specimens were collected it was felt that the patients were clearly non-schizophrenic. One, however, was tense and hypochondriacal on admission and became suspicious; it was felt

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Contribution from the Division of Biochemistry Laboratories, Department of Health, Province of Nova Scotia, Halifax, and the Nova Scotia Hospital, Dartmouth, N.S., Canada, Supported by a Federal–Provincial Mental Health Grant.

that she was probably a borderline schizophrenic. Three of these patients had recurring episodes of depression. A fifth patient had a mixture of severe tension and depression, and the sixth patient was diagnosed as a case of arteriosclerotic brain disease with depression.

Although occasionally it was possible to obtain more than one specimen of urine from a patient, as a rule therapy could not be delayed sufficiently to allow the collection of subsequent specimens. To prevent undue weighting of the results, these include only the findings for the first specimen when more than one was collected.

Complete 24-hour specimens of urine were extracted by a modification of the procedure described by Meduna and Vaichulis (1), in which the active material is adsorbed by acid-washed kaolin and eluted with dilute ammonia. The precipitate formed by the addition of acetone to the ammonia solution is collected by centrifugation and dried *in vacuo* at room temperature. Our modifications consisted in the use of "Technical Kaolin, Washed and Ignited Powder" (J. T. Baker Chemical Co.) which was not pretreated with acid but had been ground in a ball mill to prevent the formation of clumps. Secondly, the acetone precipitate was suspended in water and dried by lyophilization.

Male New Zealand white rabbits weighing approximately 2 to 4 kg. and fed Purina Rabbit Chow Checkers ad libitum were used to test the hyperglycemic action of the preparations. Extracts were administered intravenously; preparations from patients were tested at doses corresponding to the amount excreted in 75 minutes or less per kilogram of rabbit. Preparations from normal subjects were tested in doses corresponding to the amount excreted in 150 minutes. Specimens of blood were drawn from the rabbits' ears and the glucose concentration determined by the method of Nelson (5).

Results

The hyperglycemic and toxic actions of extracts obtained from the urine of 11 schizophrenic women were compared with effects induced by extracts derived from 10 normal women and six non-schizophrenic female mental patients. Each preparation was tested on two rabbits with the exception of two of the nine extracts derived from schizophrenics (SM) and one of the six extracts obtained from non-schizophrenic patients (OM), each one of which was injected into only one animal. Rabbits were injected with these extracts and specimens for blood glucose determination were removed at the times indicated in Fig. 1.

Extracts of normal urine (NM) elicited the average response shown in Curve I. Moderate hyperglycemia, not exceeding 30 mg.%, occurred approximately 30 minutes after injection and the blood glucose level returned to values within 10 mg.% of the initial concentration about one hour after reaching this peak.

SM extracts elicited changes which differed from those produced by NM preparations (Curve II). The blood glucose concentration did not reach hyperglycemic levels during the first hour but increased by an average value of approximately 60 mg.% after 3 hours; following a moderate decline the animals were still hyperglycemic at the fourth hour.

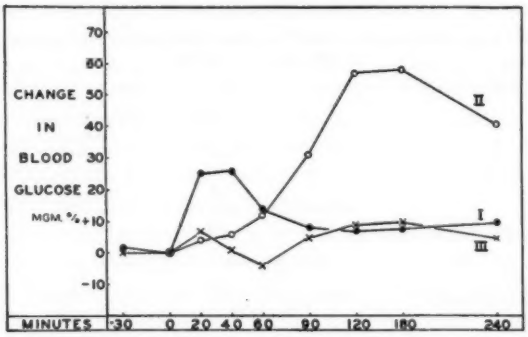

FIG. 1. Average blood glucose changes in rabbits following intravenous injection of urinary extracts.
Curve I. Normal women; 10 extracts tested on 20 rabbits.
Curve II. Schizophrenic women; 11 extracts tested on 16 rabbits.
Curve III. Other mental patients; 6 extracts tested on 11 rabbits.

Extracts obtained from the urine of non-schizophrenic mental patients did not consistently induce either early or late hyperglycemia as shown by Curve III.

The magnitude of the hyperglycemic response and the time at which maximal blood glucose changes occurred varied considerably from one extract to another, and from animal to animal when subjected to the same treatment. Statistical analysis of these findings is shown in Table I, which presents the incidence of hyperglycemia 180 minutes after injection, both in terms of the number of extracts in each group which induced blood glucose changes of 30 mg.% or more, and the number of rabbits responding in this manner.

Of the 11 SM extracts the effects of nine were studied for periods of 3 hours or more post-injection and, of these, seven were hyperglycemic. Similar changes were observed following the administration of only one of the 10 NM and one of the six OM preparations. A significantly higher incidence of hyperglycemia was induced by SM than by NM preparations, $(0.01 < P < 0.02)$. The difference between SM and OM is not significant $(0.05 < P < 0.10)$ but it should be noted that the number of OM extracts tested was too small to allow a definite conclusion.

When compared on the basis of the number of hyperglycemic rabbits in each group, the incidence of hyperglycemia is significantly higher among SM treated animals than among those receiving NM extracts. The P value of the difference between SM and OM preparations $(0.02 < P < 0.05)$ is indicative of a probable difference between these two groups.

The changes in blood glucose 180 minutes after injection were selected for comparison since it was at this time that the average response to SM preparations was maximum. Table I shows that hyperglycemia following the administration of SM was significantly higher than the blood glucose changes after NM or OM.

TABLE I

INCIDENCE OF HYPERGLYCEMIA AND TOXICITY FOLLOWING THE ADMINISTRATION OF URINARY EXTRACTS

	Normal women A	Schizophrenic patients B	Other mental patients C
No. subjects	10	11	6
Dose*/kg. rabbit	150	<75	75–150
	Hyperglycemia		
No. extracts tested	10	9	6
No. hyperglycemic extracts†	1	7	1
P	$0.01<P<0.02$‡	–	$0.05<P<0.10$§
No. rabbits tested	20	16	11
No. rabbits hyperglycemic†	1	11	2
P	<0.001‡	–	$0.02<P<0.05$§
Change in blood glucose (mg.%) at 180 min. ± S.E.	+8 ±2.90	+58 ±10.51	+7 ±6.46
P	<0.01‡	–	<0.01§
	Toxicity		
No. extracts tested	10	11	6
No. toxic extracts	0	8	0
P	<0.01‡	–	$0.01<P<0.02$§
No. rabbits tested	20	18	11
No. rabbits killed	0	11	0
P	<0.001‡	–	<0.01§

*Dose is expressed as minutes of urinary excretion corresponding to the weight of extract injected.

†Hyperglycemia taken as blood glucose increase of 30 mg.% or more above initial level, 180 minutes after injection.

‡Comparison of groups A and B.

§Comparison of groups B and C.

SM preparations also differed from normal extracts in their high degree of toxicity. Sixty per cent of the animals which received the patients' extracts died between 3 and 24 hours later. Diarrhea, cold ears, shallow rapid breathing, and limpness were the usual symptoms preceding a sudden death accompanied by convulsions and screams. On the basis of the number of lethal extracts in each group, SM preparations were significantly more toxic than either NM or OM. Whereas eight of the 11 SM extracts killed at least one rabbit, none of the animals died following the administration of any of the 10 NM or six OM preparations. When the comparison was based on the number of animals killed by each type of extract the difference was greater. Table I summarizes these results.

Discussion

The amounts of extract obtained from 24-hour specimens of urine from patients and normal subjects were about the same, the average weights and standard errors being 215 ± 32 and 220 ± 30 mg. respectively. The *P* value

of the difference was higher than 0.9, suggesting that the extracts are comparable on the basis of either weight or amount excreted per minute. In an effort to conserve SM extracts so that chemical and pharmacological properties could be studied, patients' extracts were tested at various dose levels which were, however, less than one-half the dose at which normal extracts were tested. The activity of some of the SM extracts was high enough to kill test animals when given in doses, per kilogram body weight, corresponding to less than the amount excreted in 10 minutes.

Attempts have been made to find test objects other than the rabbit which might require smaller amounts of material to demonstrate the toxic action of SM extracts. Patients' extracts were not lethal to mice when injected either intravenously or intracranially. Negative results were also found when extracts were injected into the lymph sac of frogs. When administered intravenously to rats SM extracts induced diarrhea and the animals appeared sluggish and sick; however, all 14 rats tested survived the treatment.

The findings presented confirm the observations of Meduna and Vaichulis (1) and Morgan and Pilgrim (2), who have reported that extracts of the urine of schizophrenic patients induce a greater hyperglycemic response in experimental animals than similarly prepared extracts from normal urine. The results show that the hyperglycemic responses to these two types of preparations differ not only in magnitude but also in their time relationships when the extracts are administered intravenously. Following the injection of normal extracts, maximal hyperglycemia occurs in less than 1 hour (usually in 20 to 40 minutes) and returns to normal levels 1½ hours after administration. On the other hand the patients' extracts elicit, on the average, maximum increases in blood glucose in 3 hours, and hyperglycemic levels are still found at the fourth hour. It is not possible at present to interpret the failure of both SM and OM to induce early hyperglycemia, but it should be recalled that in the case of NM this response may be due to the presence of the hypotensive agent kallikrein and that it can be inhibited by adrenolytic drugs (3).

It can be seen from the findings above that NM extracts failed to kill any of the rabbits tested. In preliminary experiments, not reported here, in which normal extracts were tested at lower doses, two out of 38 rabbits died. Meduna and Vaichulis (1) make no mention of a lethal agent in their preparations, and it may be that this effect is not observed when extracts are administered intraperitoneally. In the experiments reported above all injections were given intravenously. Morgan and Pilgrim (2) report an unspecified incidence of mortality in rats injected with crude urinary extracts from schizophrenic patients.

It appears from the observations reported that the urine of schizophrenic women contains substance(s) which frequently induce late hyperglycemia and death when injected intravenously into rabbits. When similarly prepared extracts of the urine of normal women are injected toxicity and late hyperglycemia rarely occur. The data suggest that these actions may be characteristic of the urine of acutely ill schizophrenics but do not allow any conclusions

to be drawn regarding the etiology or fundamental processes involved in schizophrenia. Further work is necessary to test the possibility that similar findings might be observed in other mental diseases or in acute disease in general.

Acknowledgments

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